

What is Claimed is:

1. A method for producing a human-like glycoprotein in a non-human eukaryotic host cell comprising the step of diminishing or depleting the activity of
5 one or more enzymes in the host cell that transfers a sugar residue to the 1,6 arm of a lipid-linked oligosaccharide structure.
2. The method of claim 1, further comprising the step of introducing into the host cell at least one glycosidase activity.
3. The method of claim 2, wherein at least one glycosidase activity is a
10 mannosidase activity.
4. The method of claim 1, further comprising producing an N-glycan.
5. The method of claim 4, wherein the N-glycan has a $\text{GlcNAcMan}_x\text{GlcNAc}_2$ structure wherein X is 3, 4 or 5.
6. The method of claim 5, further comprising the step of expressing within the
15 host cell one or more enzyme activities, selected from glycosidase and glycosyltransferase activities, to produce a $\text{GlcNAc}_2\text{Man}_3\text{GlcNAc}_2$ structure.
7. The method of claim 6, wherein the activity is selected from α -1,2 mannosidase, α -1,3 mannosidase and GnTII activities.
8. The method of claim 1, wherein at least one diminished or depleted enzyme
20 is selected from the group consisting of an enzyme having dolichyl-P-Man:Man₅GlcNAc₂-PP-dolichyl α -1,3 mannosyltransferase activity; an enzyme having dolichyl-P-Man:Man₆GlcNAc₂-PP-dolichyl α -1,2 mannosyltransferase activity and an enzyme having dolichyl-P-Man:Man₇GlcNAc₂-PP-dolichyl α -1,6 mannosyltransferase activity.

9. The method of claim 1, wherein the diminished or depleted enzyme has dolichyl-P-Man:Man₅GlcNAc₂-PP-dolichyl alpha-1,3 mannosyltransferase activity.
10. The method of claim 1, wherein the enzyme is diminished or depleted by
5 mutation of a host cell gene encoding the enzymatic activity.
11. The method of claim 10, wherein the mutation is a partial or total deletion of a host cell gene encoding the enzymatic activity.
12. The method of claim 1, wherein the glycoprotein comprises *N*-glycans having seven or fewer mannose residues.
- 10 13. The method of claim 1, wherein the glycoprotein comprises *N*-glycans having three or fewer mannose residues.
14. The method of claim 1, wherein the glycoprotein comprises one or more sugars selected from the group consisting of galactose, GlcNAc, sialic acid, and fucose.
- 15 15. The method of claim 1, wherein the glycoprotein comprises at least one oligosaccharide branch comprising the structure NeuNAc-Gal-GlcNAc-Man.
16. The method of claim 1, wherein the host is a lower eukaryotic cell.
17. The method of claim 1, wherein the host cell is selected from the group consisting of *Pichia pastoris*, *Pichia finlandica*, *Pichia trehalophila*, *Pichia*
20 *koclamae*, *Pichia membranaefaciens*, *Pichia opuntiae*, *Pichia thermotolerans*, *Pichia salictaria*, *Pichia guercuum*, *Pichia pijperi*, *Pichia stiptis*, *Pichia methanolica*, *Pichia sp.*, *Saccharomyces cerevisiae*, *Saccharomyces sp.*, *Hansenula polymorpha*, *Kluyveromyces sp.*, *Candida albicans*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae*, *Trichoderma reesei*, *Chrysosporium*

lucknowense, *Fusarium sp.*, *Fusarium gramineum*, *Fusarium venenatum* and *Neurospora crassa*.

18. The method of claim 1, wherein the host cell is further deficient in expression of initiating α -1,6 mannosyltransferase activity.

5 19. The method of claim 18, wherein the host cell is an OCH1 mutant of *P. pastoris*.

20. The method of claim 1, wherein the host cell expresses GnTI and UDP-GlcNAc transporter activities.

21. The method of claim 1, wherein the host cell expresses a UDP- or GDP-specific diphosphatase activity.

22. The method of claim 1, further comprising the step of isolating the glycoprotein from the host.

23. The method of claim 22, further comprising the step of subjecting the isolated glycoprotein to at least one further glycosylation reaction *in vitro*, subsequent to its isolation from the host.

24. The method of claim 1, further comprising the step of introducing into the host a nucleic acid molecule encoding one or more enzymes involved in the production of GlcNAcMan₃GlcNAc₂ or GlcNAc₂Man₃GlcNAc₂.

25. The method of claim 24, wherein at least one of the enzymes has mannosidase activity.

26. The method of claim 25, wherein the enzyme has an α -1,2-mannosidase activity and is derived from mouse, human, *Lepidoptera*, *Aspergillus nidulans*, *C. elegans*, *D. melanogaster*, or *Bacillus sp.*

27. The method of claim 25, wherein the enzyme has an α -1,3-mannosidase activity.
28. The method of claim 24, wherein at least one enzyme has glycosyltransferase activity.
- 5 29. The method of claim 28, wherein the glycosyltransferase activity is selected from the group consisting of GnTI and GnTII.
30. The method of claim 24, wherein at least one enzyme is localized by forming a fusion protein between a catalytic domain of the enzyme and a cellular targeting signal peptide.
- 10 31. The method of claim 30, wherein the fusion protein is encoded by at least one genetic construct formed by the in-frame ligation of a DNA fragment encoding a cellular targeting signal peptide with a DNA fragment encoding a glycosylation enzyme or catalytically active fragment thereof.
32. The method of claim 31, wherein the encoded targeting signal peptide is
15 derived from a member of the group consisting of mannosyltransferases, diphosphotases, proteases, GnT I, GnT II, GnT III, GnT IV, GnT V, GnT VI, GalT, FT, and ST.
33. The method of claim 31, wherein the catalytic domain encodes a glycosidase or glycosyltransferase that is derived from a member of the group
20 consisting of GnT I, GnT II, GnT III, GnT IV, GnT V, GnT VI, GalT, Fucosyltransferase and ST, and wherein the catalytic domain has a pH optimum within 1.4 pH units of the average pH optimum of other representative enzymes in the organelle in which the enzyme is localized, or has optimal activity at a pH between 5.1 and 8.0.

34. The method of claim 31, wherein the nucleic acid molecule encodes one or more enzymes selected from the group consisting of UDP-GlcNAc transferase, UDP-galactosyltransferase, GDP-fucosyltransferase, CMP-sialyltransferase, UDP-GlcNAc transporter, UDP-galactose transporter, GDP-fucose transporter, CMP-sialic acid transporter, and nucleotide diphosphatases.
35. The method of claim 31, wherein the host expresses GnTI and UDP-GlcNAc transporter activities.
36. The method of claim 31, wherein the host expresses a UDP- or GDP-specific diphosphatase activity.
37. The method of claim 1, further comprising the step of introducing into a host that is deficient in dolichyl-P-Man:Man5GlcNAc2-PP-dolichyl alpha-1,3 mannosyltransferase activity a nucleic acid molecule encoding one or more enzymes for production of a GlcNAcMan₄GlcNAc₂ carbohydrate structure.
38. The method of claim 1, further comprising the step of introducing into a host that is deficient in dolichyl-P-Man:Man6GlcNAc2-PP-dolichyl alpha-1,2 mannosyltransferase or dolichyl-P-Man:Man7GlcNAc2-PP-dolichyl alpha-1,6 mannosyltransferase activity a nucleic acid molecule encoding one or more enzymes for production of a GlcNAcMan₄GlcNAc₂ carbohydrate structure.
39. The method of claim 37 or 38, wherein the nucleic acid molecule encodes at least one enzyme selected from the group consisting of an α -1,2 mannosidase, UDP GlcNAc transporter and GnT1.
40. The method of claim 39, further comprising the step of introducing into the deficient host cell a nucleic acid molecule encoding an α -1,3 or an α -1,2/ α -1,3

mannosidase activity for the conversion of the GlcNAc₁Man₄GlcNAc₂ structure to a GlcNAc₁Man₃GlcNAc₂ structure.

41. The method of claim 1, further comprising the step of introducing into the host a nucleic acid molecule encoding one or more enzymes for production of a
5 GlcNAc₂Man₃GlcNAc₂ carbohydrate structure.

42. The method of claim 41, wherein at least one enzyme is GnTII.

43. The method of claim 1, further comprising the step of introducing into the host cell at least one nucleic acid molecule encoding at least one mammalian glycosylation enzyme selected from the group consisting of a glycosyltransferase,
10 fucosyltransferase, galactosyltransferase, N-acetylgalactosaminyltransferase, N-acetylglucosaminyltransferase and sulfotransferase.

44. The method of claim 1, comprising the step of transforming host cells with a DNA library to produce a genetically mixed cell population expressing at least one glycosylation enzyme derived from the library, wherein the library comprises
15 at least two different genetic constructs, at least one of which comprises a DNA fragment encoding a cellular targeting signal peptide ligated in-frame with a DNA fragment encoding a glycosylation enzyme or catalytically active fragment thereof.

45. A host cell produced by the method of claim 1 or 44.

46. A human-like glycoprotein produced by the method of claim 1 or 44.

20 47. A nucleic acid molecule comprising or consisting of at least **forty-five** consecutive nucleotide residues of Fig. 6 (*P. pastoris* *ALG 3* gene).

48. A vector comprising a nucleic acid molecule of claim 47.

49. A host cell comprising a nucleic acid molecule of claim 47.

50. A *P.pastoris* cell in which the sequences of Fig. 6 (*P. pastoris* *ALG 3* gene), are mutated whereby the glycosylation pattern of the cell is altered.
51. A method to enhance the degree of glucosylation of lipid-linked oligosaccharides comprising the step of increasing alpha-1,3 glucosyltransferase
5 activity in a host cell.
52. A method to enhance the degree of glucosylation of lipid-linked oligosaccharides comprising decreasing the substrate specificity of oligosaccharyl transferase activity in a host cell.
53. A method for producing in a non-mammalian host cell an immunoglobulin
10 polypeptide having an N-glycan comprising a bisecting GlcNAc, the method comprising the step of expressing in the host cell a GnTIII activity.
54. A non-mammalian host cell that produces an immunoglobulin having an N-glycan comprising a bisecting GlcNAc.
55. An immunoglobulin produced by the host cell of claim 54.
- 15 56. A method for producing in a non-human host cell a polypeptide having an N-glycan comprising a bisecting GlcNAc, the method comprising the step of expressing in the host cell a GnTIII activity.
57. A non-human host cell that produces a polypeptide having an N-glycan comprising a bisecting GlcNAc.
- 20 58. A polypeptide produced by the host cell of claim 57.
59. A method for producing a human-like glycoprotein in a non-human eukaryotic host cell comprising the step of diminishing or depleting from the host

cell an *alg* gene activity and introducing into the host cell at least one glycosidase activity.

60. A method for producing a human-like glycoprotein having an N-glycan comprising at least two GlcNAcs attached to a trimannose core.